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Evaluation of Polyamine and Proline Levels during Low Temperature Acclimation of Citrus[1]

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ABSTRACT

The polyamines (PA) putrescine (Put), spermidine (Spd), and spermine (Spm) were measured during 3 weeks exposure to cold hardening (15.6°C day and 4.4°C night) and nonhardening (32.2°C day and 21.1°C night) temperature regimes in three citrus cultivars: sour orange (SO) (*Citrus aurantium* L.), 'valencia' (VAL) (*Citrus sinensis* L. Osbeck), and rough lemon (RL) (*Citrus jambhiri* Lush). The changes in PA were compared to the amount of free proline, percent wood kill and percent leaf kill. A 2- to 3-fold increase in Spd concentrations were observed in hardened RL, SO, and VAL leaves compared to nonhardened leaves. Spermidine reached its highest level of approximately 200 nanomoles per gram fresh weight after 1 week of acclimation in both SO and VAL leaves, while RL spermidine content continued to increase up to the third week of acclimation. Spm levels in acclimated VAL and RL leaves increased 1- to 4-fold. However, SO leaves Spm content decreased with acclimation. Putrescine levels in SO and VAL increased 20 to 60% during the first 2 weeks of acclimation then declined after 3 weeks. RL putrescine content was not affected by cold acclimation. The data presented here provided direct relationship between increased Spd concentration and citrus cold hardiness. Free proline was 3- to 6-fold higher in acclimated than in nonacclimated trees. Results also demonstrate that in acclimated *versus* nonacclimated citrus trees the absolute amount rather than the ratio of increase in free proline is more important in predicting their ability to survive freezing stress.

In spite of an enormous amount of research, plant cold hardiness and freezing stress are still obscure phenomena (for reviews see 16, 27, 28). During late summer and early fall several environmentally induced changes take place in citrus tissues. These changes are associated with hardy plants which can undergo biochemical and physiological changes to withstand otherwise injurious temperatures (28). In hardy plants, decrease in photoperiod and day and night temperatures result in a decline in starch (30) and an increase in sucrose, and to lesser extents glucose and fructose (11, 30). Similarly, hardy plants exhibit an increase in proline, ATP, ABA, glutamic acid, ascorbic acid, and unsaturated fatty acids compared to that of nonacclimated plants (15, 25, 29). Studies have shown that a translocatable factor is synthesized during low temperature exposure which has tentatively been identified as ABA (8). To our knowledge, however, no further research has been done to try to isolate or characterize this hardening factor.

Polyamines especially Put[2] have been associated with various stress conditions (6, 7, 22, 31). Put has been shown to accumulate during chilling injury of citrus and pepper fruits (17), K^+ and Mg^{2+} deficiencies (14, 21, 22), in Cd^{2+} treated bean and oat leaves (26), at low pH (31), and during SO_2 fumigation (20). Recently, it was reported that the relative changes rather than the absolute levels of Put are more important in predicting *Phaseolus* species responses to chilling temperature (12). The increase in Put is believed to contribute to balancing the increase in anion concentration during these stress conditions. Polyamines have also been implicated in increased membrane thermostability (1, 2, 23, 24). The objectives of this study are to examine the changes in polyamines during low-temperature acclimation of several citrus cultivars and to relate the changes to their ability to withstand freezing stress.

MATERIALS AND METHODS

Plant Material. Nine-month-old trees of SO (*Citrus aurantium* L.), RL (*Citrus jambhiri* Lush.), and VAL (*Citrus sinensis* L. Osbeck) grafted on SO rootstocks were grown in 4-L plastic containers filled with peat moss, vermiculite, and perlite of equal proportions. Water and fertilizer were added according to a standard greenhouse procedure.

Chemicals. Spermidine and spermine were purchased from Calbiochem[3]; reagent grade benzoyl chloride and HPLC grade acetonitrile were purchased from J. T. Baker Chemical Co., and proline and putrescine were purchased from Sigma.

Acclimation Procedure. Thirty test plants were selected for uniform growth and general appearance from a test population of 200 plants. One-half of the test plants were placed in a walk-in chamber set at 32.2°C d and 21.1°C night temperature for 3 weeks. The other half of the plants were placed in a similar chamber set at 15.6°C d and 4.4°C night temperature for a similar period of time. Both chambers were maintained at approximately 400 $\mu E/m^2 \cdot s$ using a mixture of cool-white fluorescent and incandescent light. The relative humidity was maintained at about 60% day and night. Plants were kept under these conditions for 3 weeks. Leaf samples, 6 leaves per plant (2 from top, 2 from middle, and 2 from the bottom of each plant), 5 plants per replicate and 3 replicates in each temperature treatment were collected at the beginning of the test and at the end of each week. At the end of 3 weeks, leaf samples were also collected for proline

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[2] Abbreviations: Put, putrescine; PA, polyamines; SO, sour orange; RL, rough lemon; VAL, 'Valencia'; Spd, spermidine; Spm, spermine.

[3] Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

analysis.

Test plants were freeze tested using a standard freeze regime (29). The freeze regime began at 4.4°C for 2 h followed by a 1.1°C/h temperature drop to −6.7°C, which was maintained for 4h; temperature was then increased at a rate of 1.1°C/h to 4.4°C. After the freeze test, plants were maintained at 23°C for 4 h then were transferred to a greenhouse and kept under injury observation for 5 weeks. Plants were then rated according to percent foliage kill and stem dieback.

Polyamine Analysis. Leaf samples were weighed, rinsed several times with distilled water, and blotted dry. A total of 5 g fresh weight of leaf tissue was homogenized in 5% (v/v) $HClO_4$ to give a final concentration of 0.1 g/ml, using a polytron ® homogenizer (Brinkman Inst. Co., Div. of Syborn Corp., Westbury, NJ). The tissue extract was centrifuged for 20 min at 20,000*g* and the supernatant was used for PA analysis according to a previously described procedure (5) with some modifications. A total of 0.5 ml of the extract was added to 2 ml of 4 N NaOH and 5 μl benzoyl chloride. The mixture was vortexed for 15 s and incubated in a water bath at 35°C for 20 min. The benzoylation reaction was terminated by adding 4 ml concentrated NaCl and the benzoylated PA were extracted with 4 ml chilled diethyl ether using a vortex mixer for 20 s. The ether fraction was collected and centrifuged at 1,500*g* for 10 min at 4°C. An aliquot of 2 ml of the ether fraction was brought to dryness in a vacuum oven set at room temperature. The derivatized PA were resuspended in 200 μl chilled acetonitrile. A 10 μl aliquot was injected into a Water HPLC system (Waters Associates, Inc., Milford, MA) equipped with a UV detector set at 254 nm wavelength and a C-18 reverse phase column (4.6 mm × 250 mm). Acetonitrile/water (1:1 v/v) was used as the mobile phase in an isocratic elution.

Proline Analysis. Leaf tissues were dried in a forced-air oven set at 90°C for 24 h, and 0.2 g subsamples were soxhlet-extracted in 76% (v/v) ethanol. The ethanol extract was partitioned on a Dowex 50Wx4 (H^+) column and the amount of free proline was determined spectrophotometrically according to a previously described procedure (3).

RESULTS

Effect on Foliage and Wood Kill. Nonhardened SO, VAL, and RL kept for 3 weeks at 32.2/21.1°C day and night temperature were not able of surviving the freeze regime, as was evident by the 100% leaf and wood kill (Table I). Hardened trees kept for 3 weeks at 15.6°C/4.4°C d and night temperatures showed better survival rates than nonhardened trees. Only 20% wood kill was observed in SO, 29% in VAL and 50% wood kill in the more vigorous root stock RL. In hardened plants leaf kill was higher than wood kill in all treatments. In hardened SO about 30% leaf kill, in VAL 63%, and in RL 100% leaf kill were observed as a result of the freeze test. New foliage growth on the undamaged wood was observed sooner on RL than VAL or SO. SO and VAL are classified as moderately cold tolerant, with SO rated at slightly higher than VAL, while RL is classified as cold sensitive (28).

Table I. *Effect of Freezing Stress on Percent Wood Kill and Percent Leaf Kill in Nonhardened and Hardened Citrus Trees*

Nonhardened plants were exposed to 32.2°C d and 21.1°C night temperature and hardened plants were exposed to 15.6°C d and 4.4°C night temperature for 3 weeks. Plants were then exposed to a freeze regime of −6.7°C for 4 h. The data represent the means ± the SE of three replicates.

Citrus Cultivar	Nonhardened		Hardened	
	Wood kill	Leaf kill	Wood kill	Leaf kill
	%			
SO (*Citrus aurantium*)	100[a]	100	20 ± 5	30 ± 3
VAL (*Citrus sinensis*)	100	100	29 ± 7	63 ± 9
RL (*Citrus jambhiri*)	100	100	50 ± 10	100 ± 0

[a] Injury observations were made after the trees were held in a greenhouse for 5 weeks.

Changes in Free Proline. The concentration of free proline varied in the unhardened citrus cultivars. SO had the highest free proline 7.9 mg/g dry weight, while VAL and RL have the lowest free proline, 4.2 and 3.9 mg/g dry weight, respectively (Table II). During cold hardiness, free proline accumulated in all three cultivars tested. SO showed the highest concentration of free proline at 24.6 mg/g dry weight, and VAL showed 24.2 mg/g dry weight. The cold tender RL showed significantly less free proline, 20.0 mg/g dry weight. On a ratio basis, however, the increase in free proline in hardened RL leaves was more than 5-fold higher than in nonhardened leaves. In VAL, a moderately cold tolerant cultivar, the rate of increase in free proline in hardened leaves was 5.8-fold and in SO, a cold tolerant cultivar, it was 3.1-fold higher than in nonhardened leaves. In general, free proline accumulated relative to the degree of freeze tolerance; however, the rate of free proline accumulation was inversely proportional to the degree of freeze tolerance. Thus, in citrus leaves the absolute amount rather than the rate of increase in free proline concentration is a more reliable marker for predicting cold hardiness.

Changes in PA titers. HPLC analysis of PA titers in leaves of hardened and nonhardened citrus showed variable differences in their concentrations (Table III). In nonhardened SO, RL, and VAL the average Put concentrations during the 3 weeks at 32.2°C d and 21.1°C night temperatures were 42, 40, and 29 nmol/g fresh weight, respectively. In contrast, Spm concentrations in nonhardened VAL was (229 nmol/g fresh weight) 2.3-fold higher than SO (97 nmol/g fresh weight), and approximately 10-fold higher than RL (24 nmol/g fresh weight). Examination of Put and Spm levels during the 3 weeks at the nonhardening temperature regime showed no significant change in their concentrations, except in few cases which may have been due to a slight water stress.

In hardened SO and VAL leaves, Put concentrations during the first 2 weeks exposure to 15.6°C d and 4.4°C night temperatures showed an increase of up to 60%, then the concentrations declined to about the control level after 3 weeks of acclimation (Table III). Higher Spm levels were observed in acclimated VAL and to a lesser extent in RL leaves. In comparison, SO showed no significant change in Spm concentration during acclimation. Similar to nonhardened leaves, hardened VAL leaves continued

Table II. *Changes in Free Proline in Nonhardened and Hardened Citrus Leaves*

Temperature regimes were similar to those outlined in Table I except leaves were collected for proline analysis prior to plants exposure to the freeze regime. The data represent the means ± the SE of three replicates.

Citrus Cultivar	Nonhardened	Hardened
	mg/g dry wt	
SO (*Citrus aurantium*)	7.9 ± 0.5	24.6 ± 0.4 (3.1)[a]
VAL (*Citrus sinensis*)	4.2 ± 0.1	24.2 ± 0.5 (5.8)
RL (*Citrus jambhiri*)	3.9 ± 0.7	20.0 ± 1.0 (5.1)

[a] The data in parentheses represent the ratio of free proline content in hardened plants compared to nonhardened plants.

Table III. *Effect of Nonhardening and Hardening Temperature Regimes on Putrescine and Spermine Content of Citrus Leaves*

Temperature regimes were similar to those outlined in Table I. The data represent the means ± the SE of three replicates.

Citrus Cultivar	Acclimation Period	Nonhardened		Hardened	
		Put	Spm	Put	Spm
	weeks	*nmol/g fresh wt*			
SO (*Citrus aurantium*)	0	41 ± 8	75 ± 7	43 ± 5	45 ± 6
	1	41 ± 7	113 ± 9	53 ± 9	39 ± 4
	2	38 ± 5	82 ± 2	61 ± 4	48 ± 6
	3	47 ± 16	119 ± 23	51 ± 12	43 ± 13
VAL (*Citrus sinensis*)	0	29 ± 8	210 ± 20	26 ± 8	258 ± 3
	1	28 ± 6	182 ± 9	33 ± 5	303 ± 20
	2	32 ± 4	272 ± 27	41 ± 2	349 ± 48
	3	27 ± 3	252 ± 31	30 ± 1	237 ± 19
RL (*Citrus jambhiri*)	0	28 ± 4	14 ± 2	32 ± 1	15 ± 2
	1	54 ± 7	28 ± 5	37 ± 3	42 ± 2
	2	48 ± 7	38 ± 10	30 ± 1	52 ± 4
	3	30 ± 5	17 ± 2	34 ± 4	75 ± 9

to show higher Spm than SO or RL.

Spermidine levels in all three cultivars tested (Figs. 1, 2, and 3) showed the most significant and uniform change compared to Put and Spm. In hardened SO and VAL leaves, Spd reached its maximum level of 200 nmol/g fresh weight after only 1 week of acclimation. Cold hardened RL continued to show an increase in Spd content up to the third week of acclimation, a possibility that RL leaves have not reached their maximum hardening level. Spermidine concentrations in nonhardened SO, VAL, and RL remained unchanged throughout the test period.

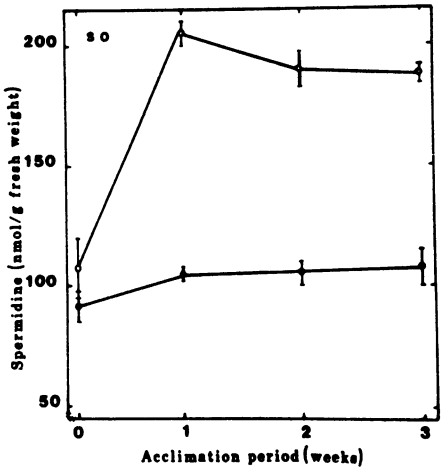

FIG. 1. Leaf spermidine content of nonhardened (●) and hardened (○) SO trees. Details of experimental conditions are described in the text. The data represent the means ± the SE (bars) of three replicates.

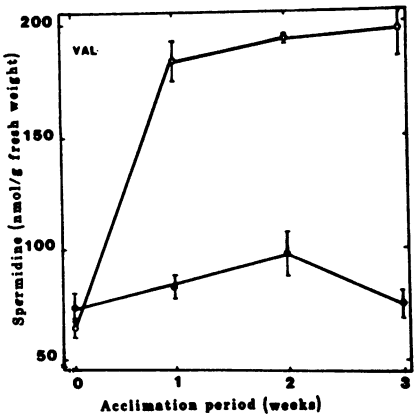

FIG. 2. Leaf spermidine content of nonhardened (●) and hardened (○) VAL trees. Details of experimental conditions are described in the text. The data represent the means ± the SE (bars) of three replicates.

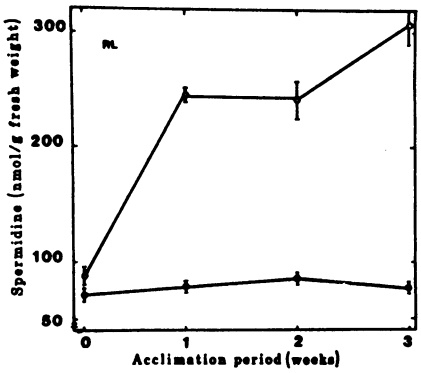

FIG. 3. Leaf Spd content of nonhardened (●) and hardened (○) RL trees. Details of experimental conditions are described in the text. The data represent the means ± the SE (bars) of three replicates.

DISCUSSION

Polyamines have been associated with various stress conditions (17, 21, 23, 31). We have formulated this study to examine the changes in PA titers during low-temperature acclimation of three citrus cultivars and to relate the changes to their ability to survive freezing stress. The data presented clearly demonstrate that citrus trees responded to low temperature acclimation with a uniform and substantial increase in proline and Spd, but less uniform increase in Put and Spm. The bases for stress induced changes in these compounds are at the present not clearly understood. Several hypotheses for stress induced accumulation of PA have been presented (7). One hypothesis suggests that the increase in PA synthesis are in response to acidification of the cytoplasm as a result of anion/cation imbalance (7, 22). Initial investigation with citrus showed a characteristic increase in phospholipids and other polar compounds during citrus cold hardiness (28). The increase in these polar compounds could have provided the basis for the increase in PA titers. Another hypothesis suggests that polyamines increase during stress may have a direct role in maintaining cell membrane thermostability against changes in fluidity and solute leakage (23). Application of 1 to 10 mM Put

or Spd protected isolated protoplasts against lysis and reduced macromolecules breakdown (2, 9).

The most important observation in this study is that Spd titer accumulation was more significant than either Put or Spm. The increase in Spd titer followed a moderate rise in Put in both VAL and SO trees. In RL, Spd titer accumulation was induced without a proceeding increase in Put titer (Table III). Flores *et al.* (7) reported a 30 to 50% increase in Spd concentration in osmotically stressed oat seedlings followed an initial surge in Put titer. Previous reports have indicated that Spd accumulation contributed significantly toward maintaining cell membrane integrity (12, 23, 24). Using specific inhibitors of Spd synthase, it was concluded that Spd, rather than Put, is required for growth and development (4). The physiological importance of Spd has been recognized in studies on the senescence of oat leaves and cell suspension cultures (13, 18).

Using mature mung-bean hypocotyls, it was observed that Spd was always the main bound form of PA, while Put was the main free form of PA (10). Based on the previous observation, the *de novo* increase in Spd titer in cold hardened citrus leaves may have resulted from a shift in the ratio of bound to free Spd, in addition to a possible stimulation of Spd synthase. The role of Spd in acclimation to low temperature may resemble that of long chain alkyldiamines which have been patented for protecting crops against frost damage (19).

As in previous studies (29), free proline accumulated 3- to 6-fold higher in acclimated than in nonacclimated tissues. Higher proline was significantly correlated with the ability of the test plants surviving the freeze damage. Increase in proline during environmental stress was positively correlated with stress tolerance in other systems (15). Our results demonstrate that the absolute amount rather than the percent increase in free proline is more important in predicting the ability of the plants to survive the freezing stress.

The increase in Spd, proline, and other compounds reported here and elsewhere during cold acclimation may collectively contribute to maintain tissue viability during freezing stress. Such contributions may include: osmoregulation, protection of cellular membranes, and enzymes, and/or conservation of energy for post stress growth.

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